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Application of Confocal Laser Scanning Microscopy in Characterization of Chemical Enhancers in Drug-in-Adhesive Transdermal Patches

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ABSTRACT The purpose of this study was to evaluate the application of confocal laser scanning microscopy (CLSM) in the examination of the embedment and the release characteristics of chemical permeation enhancers from transdermal drug delivery systems (TDDSs) of the "drug-in-adhesive" type. The enhancer lauric acid and a lauric acid fluorescing probe of the Bodipy type were incorporated into TDDSs consisting of an acrylic, a polyisobutylene, or a silicone polymer adhesive. Three-dimensional confocal images of the distribution were obtained before and during release into an aqueous medium. The images showed that the lauric acid fluorescing probe was homogeneously embedded in all the adhesives except for 1 polyisobutylene. The release profiles and release rate constants of the lauric acid fluorescing probe were consistent with data from a release study of lauric acid performed using conventional measurements of the released amounts. This indicated that lauric acid was distributed in a homogeneous manner. Furthermore, it was possible to illustrate the mechanics of the diffusion process inside the TDDS and compare these patterns with theoretically drawn profiles, based on Fick's law of diffusion. CLSM was demonstrated to be an excellent tool to study how enhancers are incorporated and diffuse into a TDDS.

KEYWORDS: confocal laser scanning, microscopy, chemical enhancers, diffusion, drug-in-adhesive patches, release mechanism

INTRODUCTION

Using confocal laser scanning microscopy (CLSM), it is possible with a high degree of precision to locate and study transport phenomena of fluorescent chemical substances in different materials. This technique is often used in evaluation of biological phenomena and transport studies through various biological membranes, eg, the intestine [1] and the skin [2]. However, until now only a few studies using CLSM have focused on the application of technical pharmaceutical aspects; eg, Cutts et al have evaluated controlled-release dosage forms (pellets) [3], Lamprecht et al have characterized microcapsules [4], and Peltonen et al have reported on surface structures of tablets [5].

In a previous study, we focused on the release kinetics of chemical permeation enhancers from a transdermal drug delivery system (TDDS) [6]. We used a TDDS of the drug-in-adhesive type where the enhancer was directly incorporated in the adhesive layer. However, only a little is known about how the enhancers behave in the adhesive. The release study showed that most of the enhancers released within a few hours, and it was speculated that the enhancers might be nonhomogeneously incorporated in the adhesive [6].

The purpose of this study was to evaluate whether CLSM could be used to examine the embedment and release kinetic of the enhancer lauric acid from TDDSs consisting of 3 different types of adhesive polymers: an acrylic, a polyisobutylene, and a silicone. No active drug substances were used in this study. Because lauric acid does not have fluorescing properties, a fluorescent probe of the Bodipy type was added to lauric acid. The labeled probe was evaluated as to whether the observed release rate of the lauric acid fluorescing probe reflected the release of lauric acid or could be due to the fact that the Bodipy probe followed the transport of any compound. This was carried out with an additional experiment where the lauric acid fluorescing probe was incorporated with 2 other enhancers, N-methyl-2-pyrrolidone (NMP) and Azone. These enhancers were known to have significantly different release characteristics and physicochemical properties than lauric acid [6].

Furthermore, the diffusion mechanics of the lauric acid fluorescing probe inside the TDDS were studied and compared to theoretically calculated curves obtained using Laplace transformations and Spiegel inversions developed by Hadgraft [7] and Crank [8] from Fick's law of diffusion.

MATERIALS AND METHODS

Materials

A fluorescent probe for lauric acid, D-3826, C10-Bodipy 500/510 C3-probe (5-decyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-propionic acid), MW = 404.31 g/mol [9], was obtained from Molecular Probes (Eugene, OR). The probe has a similar structure to lauric acid but has a fluorescing group near the acid head. Lauric acid was obtained from Merck (Darmstadt, Germany). Azone was a gift from Durham Pharmaceuticals (Durham, NC). NMP was from Fluka (Buchs, Switzerland). Durotak 387

2516 (acrylic) and NSC-58 (polyisobutylene) transdermal polymer adhesives were gifts from National Starch and Chemical (NSC, Zutphen, The Netherlands). The silicone adhesive MD7-4602 was obtained from Dow Corning (DC, Coventry, UK). Oppanol B100/B150, a blend of polyisobutylene polymers, was obtained from BASF AG (Ludwigshafen, Germany). Scotch Pak release liner 1022 was obtained from 3M Medica (Borken, Germany). The Rexam release liner was from Rexam release (Chicago, IL). All chemicals used were of the highest possible purity (> 99%), obtained from commercial sources and used as supplied.

Preparation of Patches

The formulations in Table 1 were produced as TDDSs using the following method. The individual enhancers (ie, lauric acid, Azone, NMP) and the fluorescent probe were dissolved in methanol and mixed. The methanol was allowed to evaporate, and an organic solution of adhesive polymer was added. After mixing on a Rotamixer RK 20-VS (Heto-Holten A/S, Allerød, Denmark) at 10 rpm for 2 hours, the blend was casted onto a release liner using a modified Laboratory Drawdown Coater LC 100 from ChemInstruments (Mentor, OH). The Rexam release liner (silicopolymer coated) was used for the acrylic and the polyisobutylene adhesives. For the silicone adhesive, the Scotch Pak 1022 release liner was used (fluoropolymer coated). Using a LUT 6050 oven with airflow from Heraeus Instruments (Newtown, CT) for 10 minutes at 40°C, the solvent from the adhesive was allowed to evaporate. Because NMP was known to evaporate from the adhesive in this process, this laminate was produced with a surplus of enhancer using a validated method [6]. The concentration of the lauric acid fluorescing probe in all TDDSs was 50 mM, and for lauric acid and the lauric acid fluorescing probe the concentration was in total 7.5% (wt/wt).

Table 1 - Formulations Examined*

No.	Enhancer	Adhesive
1	Lauric acid	Acrylic (NSC)
2	Lauric acid	Pib-58 (NSC)
3	Lauric acid	Oppanol Pib (BASF)
4	Lauric acid	Silicone (DC)
5	Azone	Acrylic (NSC)
6	NMP	Acrylic (NSC)
7	None	Acrylic (NSC)

***All formulations were added a lauric acid fluorescent probe. NMP indicates N-methyl-2-pyrrolidone and Pib indicates PolyIsoButylene. Manufactures are shown in brackets. DC indicates, Dow Corning; NSC, National Starch Chemical**

CLSM

The CLSM used was a Zeiss LSM 510 with motorized Axiovert 100 microscope (both Carl Zeiss, Jena Germany). An argon laser was used, for excitation at 458 nm. Sections of the TDDS patches (with an area of 1 cm²) were punched out and applied in the bottom of a diffusion chamber with the release side facing upward. It was checked that no large air bubbles were present in the examined area of the patch, as this would scatter the light. Using a Scan/Neofluar 25X objective (505 nm filter, 66 µm pinhole) from Carl Zeiss, a series of 100 images in the XY plane were made, each sized 368.5 µm * 368.5 µm and representing a slab of 1.00 µm (Figure 1). Using software (Zeiss LSM, version 2.30.011), a 3-dimensional image (XYZ space) was created, sized 368.5 µm * 368.5 µm * 100 µm, with an image resolution of 1024 * 1024 pixels, meaning that every pixel represented a square of 0.36 µm * 0.36 µm (XY) and in 1.00 µm height (Z) of the adhesive. Every pixel represents a value of intensity, I, from 0 to 255, where 0 is no fluorescence detected and 255 is full saturation; no series were made with values starting above 230. This procedure was repeated using a C-Apochromat 63X (Carl Zeiss) objective for higher resolution (but smaller image section of the patch). These higher-resolution confocal images showed the distribution of the fluorescence (which reflects the distribution of the fluorescent lauric acid probe), with the pixel size representing a 0.14 µm * 0.14 µm area.

Evaluation of the release of the lauric acid fluorescing probe was then made with the 25X objective. To the diffusion chamber (with donor compartment on bottom and receiver compartment on top) 4 mL of receptor fluid was added; then the chamber was closed with a cover. The release from the TDDS to the overlying receptor fluid was followed by measuring the change in fluorescence in the TDDS with predetermined time intervals. The diffusion was followed in 10 hours, but the frequency of obtaining images was highest in the initial part. The time intervals are shown in Figure 2. The receptor fluid was stirred and consisted of a 0.05 M phosphate buffer with 4% polysorbate 80, as this was known to secure sink condition during the assay. All the described measurements were done on the TDDS formulations shown in Table 1. Furthermore, fluorescence from TDDSs without the lauric acid fluorescing probe incorporated was examined as control (placebo). The extent of photobleaching of the lauric acid fluorescing probe was quantified. This was done by an assay similar to the one described above but with a series of pictures obtained with no time intervals. The reduction in the fluorescent properties of the probe by the laser (photobleaching) could then easily be quantified. For each picture obtained, the fluorescent properties reduced 2% to 4.5% depending on the formulation of the TDDS.

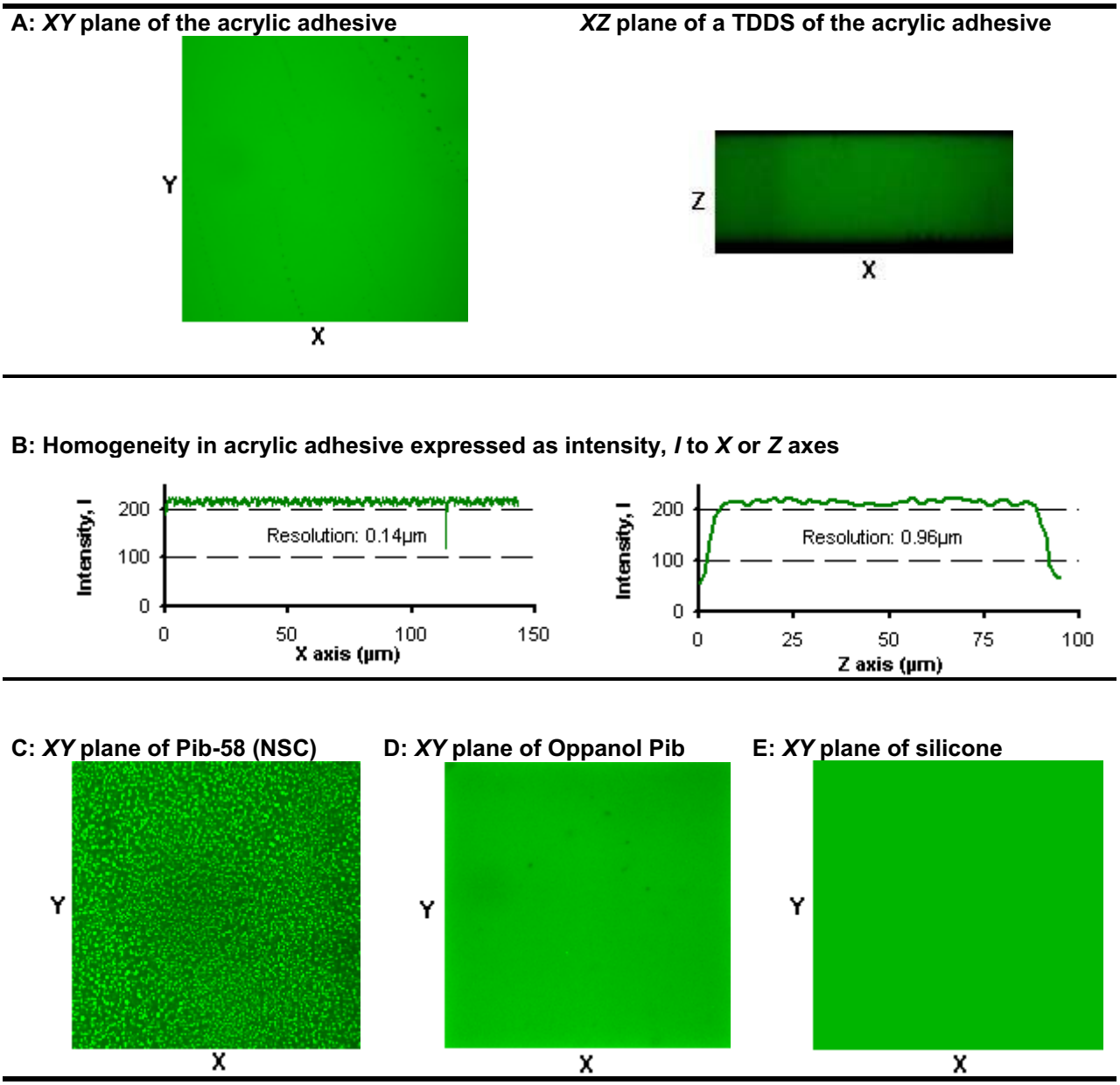


Figure 1- Images evaluating homogeneity of lauric acid fluorescent probe in the adhesives (formulations 1-4). A is the acrylic adhesive, C the NSC-Pib adhesive, D the Oppanol Pib (BASF) adhesive and E the silicone adhesive.* A continuous green colour indicates homogeneity. The intensity of each pixel in each axis is shown graphically in B for the acrylic adhesive.

*** Pib indicates polyisobutylene; NSC, National Starch Chemical, TDDS, transdermal drug delivery system.**

Statistical analysis

Student t test was used to compare the release rate constants calculated in the study with constants calculated in a previous study where release was measured by conventional methods. The significance level was $P \geq 0.95$.

RESULTS

Homogeneity

In Figure 1, images of the TDDSs with lauric acid and the fluorescent probe incorporated are shown. A is the *XY* plane in the center of the TDDS of the acrylic adhesive and the *XZ* plane of the same TDDS composed by stacking 100 "A" pictures and observing

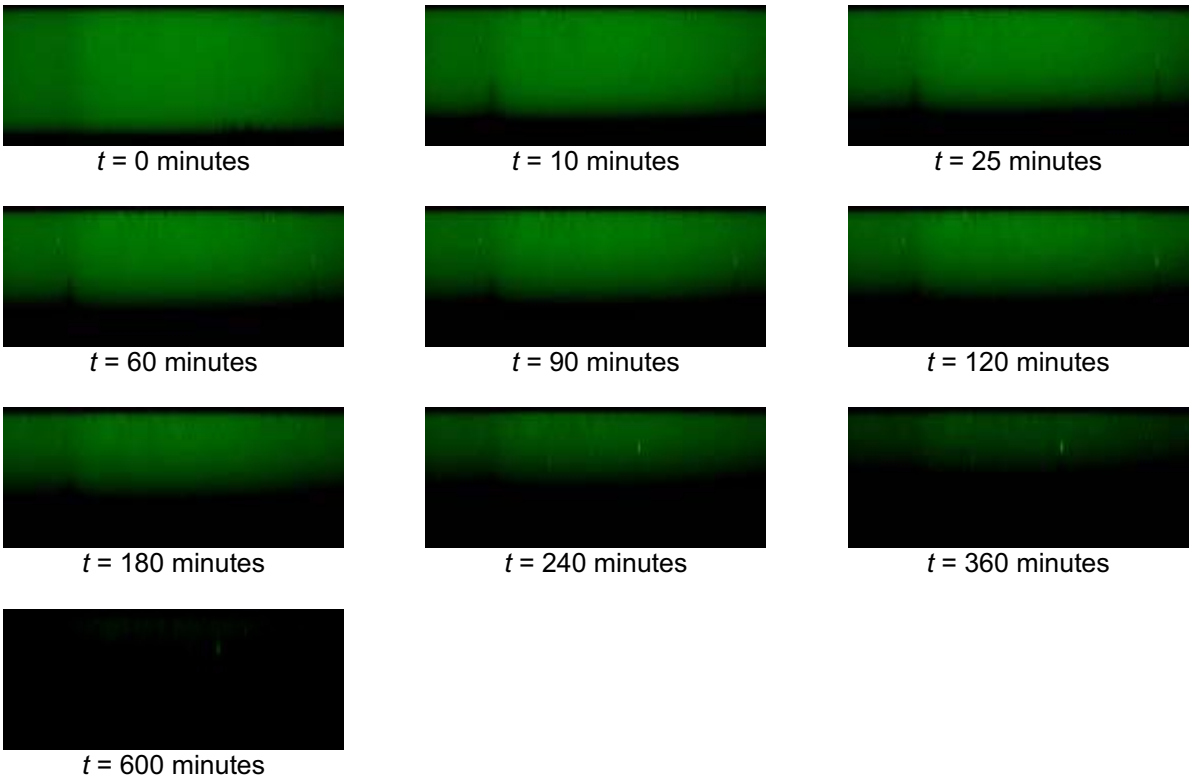


Figure 2 - Images in the *XZ* plane showing the release of lauric acid fluorescent probe from the TDDS of the acrylic adhesive (formulation 1).*

***Because the CLSM turns the images upside down, the receptor medium in these images appears to be underneath the TDDS, while in the study the lauric acid fluorescent probe released up into the receptor medium. CLSM indicates confocal laser scanning microscopy; TDDS, transdermal drug delivery system.**

from the side using the software. In the TDDS of the acrylic (Figure 1A) there is a continuous green color throughout the TDDS, disrupted only by small air bubbles scattering some of the light. This is consistent with the claim that the lauric acid fluorescent probe is homogeneously embedded in the polymeric material. This is illustrated graphically in Figure 1B for the acrylic adhesive (intensity, *I*, is plotted against distance on the *X* and *Z* axes). All formulations, summarized in Table 1, showed a similar pattern (shown for the silicone in Figure 1E). The polyisobutylene NSC-58 adhesive (Figure 1C) was an exception, as it showed fluorescence in discrete, almost porous patterns. However, the Oppanol polyisobutylene from BASF did not (Figure 1D).

Release Kinetic of Enhancer

Using Fick's law of diffusion, T Higuchi and WI Higuchi showed that the drug release from a topical product in which the drug is initially uniformly dissolved can be described by the following equation [10] :

$$Q = hC_0\left[1 - \frac{8}{\pi^2}\sum_{m=0}^{\infty}\frac{1}{(2m+1)^2}\exp\left(\frac{-D(2m+1)^2\pi^2 t}{4h^2}\right)\right] \quad (1)$$

where Q is the cumulative amount of drug released, t is time, h thickness of the adhesive layer, C0 initial drug concentration in the matrix, and D the diffusion coefficient. However, for most practical applications a simplified form of this equation may be used when less than 60% is released [10-11] :

$$R = 200\left(\frac{Dt}{\pi h^2}\right)^{1/2} \approx k * \sqrt{t} \quad (2)$$

where R is the percent of drug released and k is a release rate constant. We have previously shown that the release of enhancers from TDDSs of the drug-in-adhesive type follows the theories deduced by T. Higuchi: straight lines are obtained when fraction released (R) is plotted against the square root of time (6). In Figure 2, images from the XZ plane during release of lauric acid fluorescent probe from an acrylic adhesive

are shown. The images reveal a depletion of lauric acid fluorescent probe as time proceeds. A similar pattern was observed for all formulations in Table 1. However, the release study was not conducted for formulation 2 (with NSC-58 polyisobutylene) because it was not homogeneous, as described above.

The green color in each pixel is defined by an intensity from 0 to 255. Integration on the intensity of fluorescence in each image describes how much lauric acid fluorescent probe is left in the patch. The effect of photobleaching was preliminarily described. The reduction in fluorescence due to diffusion was mathematically corrected for the photobleaching by simple subtraction because it was known how much (in percent) the fluorescence is reduced by obtaining each picture. The reduction in fluorescence due to photobleaching was much less than the reduction due to diffusion. Release profiles could then be obtained and transformed to square root according to equation 2. The formulations 1, 3, and 4 showed straight lines, as in the example shown in Figure 3. From the slopes, release rate constants were calculated, and in Table 2 they are compared to data from a previous release study where the release was measured directly by conventional quantitative analytical methods. No significant difference was observed. This is also indicated in Figure 3 where the profile measured using conventional methodology is added. To reveal whether the lauric acid fluorescing probe just followed the transport of matter, the release from formulations 5, 6, and 7 was measured. When the lauric acid fluorescent probe was embedded with 2 enhancers with higher and slower release kinetics, Azone and NMP, respectively, no steady state release profiles similar to the conventional method were observed. In the study where release was measured by conventional methods, these enhancers had shown steady state profiles. Therefore, calculation of k for the NMP, Azone, and "no enhancer" formulations in this study was not done, as the calculations would not be valid.

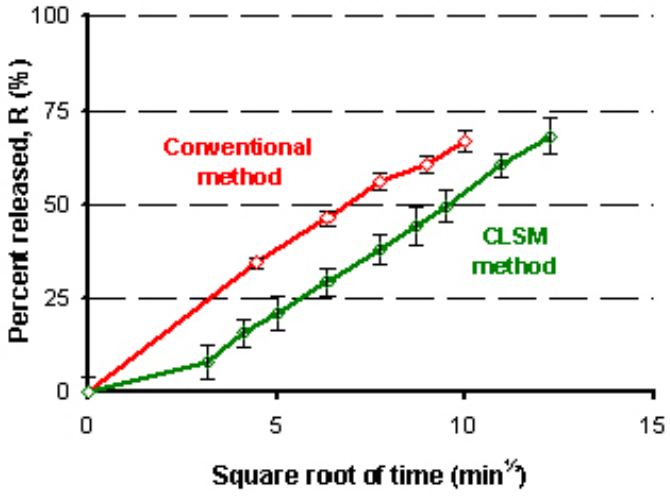


Figure 3 - Mean release profiles (n = 3) from a TDDS of acrylic adhesive transformed to the square root of t.*

***The green line is the lauric acid fluorescent probe (formulation 1) measured by the CLSM method, and the red line is the release of lauric acid measured by the conventional method (6). CLSM indicates confocal laser scanning microscopy; TDDS, transdermal drug delivery system.**

Diffusion mechanics inside the TDDS

The images in Figure 2 show the release of the lauric acid fluorescent probe from the patch (the CLSM shows the images upside down). During the release study, an increasing gradient in concentration of the lauric acid fluorescing probe could be observed in the patch. In the XZ scan, 5 specific levels of depth in the patch were defined: 10%, 30%, 50%, 70%, and 90% from the backing side. For the specific time intervals, the relative intensity for each point was plotted, as shown in Figure 4 for the lauric acid fluorescent probe in the acrylic adhesive. Near the release side (X = 90), the lauric acid fluorescent probe was quickly removed. In the other points, X = 70, 50, 30, and 10, the release rates were lower in the consecutive order.

As shown by Hadgraft, illustrations of the depletion of a diffusing molecule inside the vehicle based on Fick's law of diffusion can be mathematically modeled using Laplace transformations and Spiegel inversions [7] . Using diffusion theories similar to T. Higuchi and W.I. Higuchi (Equation 1), Hadgraft developed the following equation to describe the concentration profile of a diffusant inside a pharmaceutical vehicle:

$$\frac{C_{x,t}}{C_0} = \frac{-4}{\pi}\sum_{n=1}^{\infty}\frac{(-1)^n}{(2n-1)}\exp\left(\frac{(2n-1)^2\pi^2}{-4}\times\frac{D^2t^2}{h^4}\right)\cos\left(\frac{(2n-1)\pi}{2}\times\frac{x}{h}\right) \quad (3)$$

where Cx,t is the concentration at X, the distance in the patch at t, the time. The theoretical concentration profiles

inside the TDDS were drawn at different time intervals using the mathematical software Maple 6 from Waterloo Maple (Ontario, Canada). In Figure 5, the profiles are compared to profiles obtained from the images. Generally, there was good agreement between the theoretical and measured profiles. However, some differences could be observed; most important, the concentration of the lauric acid fluorescent probe was reduced faster than predicted at the release side of the patch.

Table 2 - Mean Release Rate Constants, *k* (in Percentage/Square Root of *t*), and RSDs (SD/k * 100, in Percent) From CLSM and Conventional Performed Release Studies Where *R* Is the Percentage of Drug Released (*n* = 3 in All Measurements)*

Formu-lation	CLSM Study		Conventional Release Study	
	k	RSD	*k*	RSD
1	6.5	(3.0)	7.3	(3.9)
2	—†	—	3.2	(5.6)
3	3.0	(2.5)	—§	—
4	6.8	(2.7)	6.9	(0.2)
5	—‡	—	3.1	(1.4)
6	—‡	—	12.2	(3.3)
7	—‡	—	—§	—

*CLSM indicates confocal laser scanning microscopy; RSD, relative standard deviation.
†Not determinable in this study due to inhomogeneous embedment of enhancer.
‡Not determinable in this CLSM study due to non-Fickian behavior in release profile.
§Not determined in study using conventional methods.

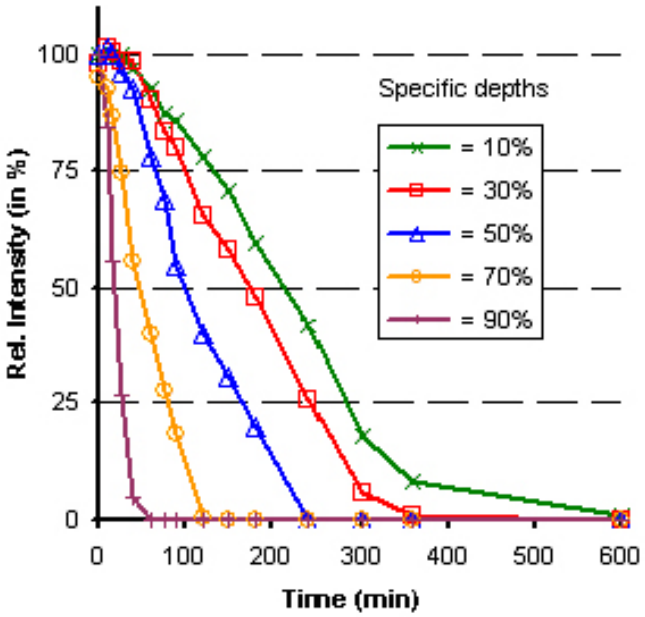
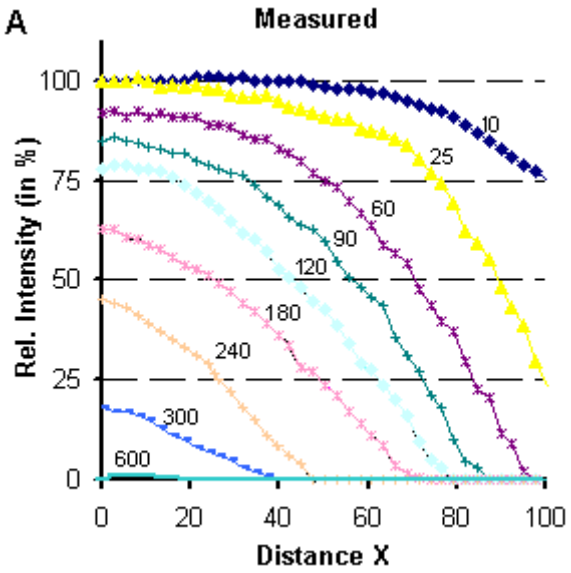


Figure 4 - Depletion of the lauric acid fluorescent probe from the acrylic adhesive TDDS (formulation 1).*

***Intensity at *t* = 0 is set to 100%, and relative intensities (*Y* axis) are plotted to time (*X* axis). *X* = 10%, etc, are the depths of the TDDS. *X* = 0% is at the backing film, whereas *X* = 100% is at the release side. TDDS indicates transdermal drug delivery system.**



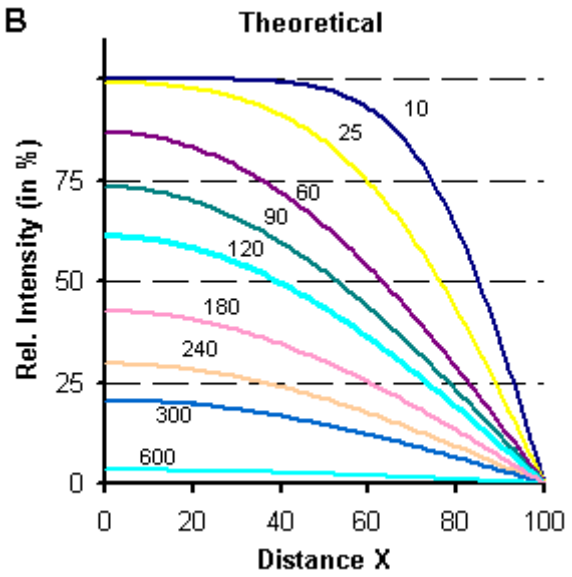


Figure 5 - Diffusion mechanics inside the TDDS of acrylic adhesive (formulation 1), relative to initial concentration.*

***The profiles in (A) are calculated from the CLSM images, and the profiles in (B) are calculated theoretically from Equation 3. Distance *X* indicates relative depth in the patch, *X* = 0 at backing and *X* = 100 at the release side of the patch. The numbers 10, 25, etc, indicate sample times in minutes. CLSM indicates confocal laser scanning microscopy; TDDS, transdermal drug delivery system.**

DISCUSSION

The objective of this study was to evaluate whether the CLSM technique could be used to evaluate how chemical enhancers behave in TDDSs of the drug-in-adhesive type. Three-dimensional images of the TDDS, showing the lauric acid fluorescent probe in the adhesive, were obtained using the CLSM technique. However, various conditions have to be fulfilled in order for this technique to be used in a scientifically valid manner. The adhesive in the TDDS needs to be at least semitransparent, and the examined enhancer needs to have fluorescent properties itself or be compatible with a suitable fluorescent probe with similar physicochemical characteristics as the enhancer. Furthermore, in quantitative experiments it is necessary to quantify the extent of photobleaching and make mathematical corrections in data handling.

The preparation of a TDDS consists of several steps: blending the components, mixing them into the adhesive polymer solution, casting on the release liner, and drying, where the organic solvent from the adhesive solution is allowed to evaporate. In a previously reported release study, the enhancers were shown to release relatively fast [6]. Given that many of the enhancers are highly soluble in organic solvents and have surfactant properties, it could be speculated that the high release rate might be due to an inhomogeneous incorporation of the enhancers in the adhesive (eg, discrete areas or a gradient of enhancer in the Z axis could be formed in the adhesive slab). Furthermore, a migration to the adhesive-backing or adhesive-release-liner interfaces might take place.

Using the highest resolution on the CLSM apparatus, this study showed images with a pixel size of 0.14 µm * 0.14 µm; the images indicated that in this scale, the lauric acid fluorescent probe was homogeneously embedded in most of the examined adhesives. This was graphically shown in Figure 1's horizontal lines for the X, Y, and Z axes, indicating that no large discrete areas or gradient of the lauric acid fluorescent probe was observed. However, for one polyisobutylene adhesive, this pattern was not observed. The polyisobutylene, NSC-58, indicated that the probe was embedded in porous structures. NSC-58 is a commercial ready-to-use polyisobutylene adhesive with good adhesive properties, partly due to an adhesive tackifying agent. It is reasonable to speculate that this tackifying agent might bind to lauric acid or the fluorescent probe; the other examined polyisobutylene, Oppanol (BASF), which consists of only polyisobutylene and solvent, did not show this effect.

The release of the lauric acid fluorescent probe from the adhesives was studied and the results were compared to the results from the release study of lauric acid performed using conventional methods. The observed release profiles and the calculated release rate constants were consistent with previous results (Table 2). When the lauric acid fluorescent probe was incorporated in the adhesive with enhancers with different physicochemical properties and release characteristics than lauric acid, neither profiles nor release rates were observed resembling the profiles previously determined for these compounds [6]. These results support the conclusion that the lauric acid fluorescent probe is incorporated in the adhesive in a similar manner to the incorporation of lauric acid, as the probe did not just follow the transport for any compounds.

Using the CLSM technique, we could follow the diffusion of the lauric acid fluorescent probe inside the TDDS during the release study. The profiles in Figure 4 show that focusing at specific depths in the patch there was a linear relationship of concentration versus time over a broad time frame. The total released amount of lauric acid fluorescent probe to the receptor media followed a square root of time relationship (Figure 3), as deduced by Equation 3. Profiles from Figure 5A illustrating the concentrations of the lauric acid fluorescent probe at different levels of depth show how the lauric acid fluorescent probe released from the patch at different time intervals. When these profiles are compared to theoretically calculated profiles in Figure 5B, it is clear that the overall pattern is the same. However, there are some differences: eg, at the start a equilibrium may not be reached and during the study the amount of lauric

acid fluorescent probe at the release side in the TDDS is reduced faster than predicted. A reason for this might be that during the release study not only does lauric acid (and the lauric acid fluorescent probe) diffuse out of the TDDS to the receptor media, but water from the media may also diffuse into the TDDS. An intrusion of water may lower the diffusion coefficient of lauric acid and the fluorescent probe on the release side of the TDDS, facilitating a release that is relatively faster than predicted.

In conclusion, the CLSM technique is effective for examining the behavior of an enhancer TDDS of the drug-in-adhesive type. It was possible to obtain images indicating a homogeneous incorporation of the lauric acid fluorescent probe in the adhesive and to measure release out of the TDDS and diffusion inside the TDDS. The release characteristics measured for the probe were similar to the characteristics for lauric acid measured using conventional methods. The diffusion inside the TDDS showed good correspondence to the theoretically calculated profiles. However, the process showed slight differences from the predicted pattern, especially at the release side, which was possibly due to absorption of water in the TDDS during the study. This technique may be very useful in other pharmaceutical applications or vehicles other than TDDSs. However, some precautions need to be considered (eg, transparency of vehicle, extent of photobleaching, choice of fluorescent probe if the substance of interest does not have fluorescent properties). Furthermore, the CLSM apparatus is quite expensive, which may limit the use of this new technique.

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